Novel Mutations in the *LAMC2* Gene in Non-Herlitz Junctional Epidermolysis Bullosa: Effects on Laminin-5 Assembly, Secretion, and Deposition

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Laminin-5 is the major adhesion ligand of epithelial cells. Mutations in the three genes (*LAMA3*, *LAMB3*, *LAMC2*) encoding the laminin-5 chains cause junctional epidermolysis bullosa, a clinically and genetically heterogeneous blistering skin disease. Here, we describe a non-Herlitz junctional epidermolysis bullosa patient, compound heterozygote for two novel mutations affecting the *LAMC2* gene. The mutation in the paternal allele is a *de novo* splice site mutation (522-1G→A) that results in in-frame skipping of exon 4 and synthesis of a mutated γ2 polypeptide (γ2Δ4) carrying a 33 amino acid deletion within the N-terminal domain V. The maternal mutation is a one base pair insertion (3511insA) in the 3′ terminal exon of *LAMC2* resulting in a frameshift and a premature termination codon. Mutation 3511insA is predicted to lead to the synthesis of a γ2 polypeptide (γ2t) disrupted in its α-helical C-terminal structure and truncated of the last 25 amino acids. Keratinocytes isolated from the patient's skin showed a markedly decreased level of γ2 chain mRNA and secreted scant amounts of laminin-5, which undergoes physiologic proteolytic processing. To investigate the biologic function of the laminin-5 molecules synthesized by the patient, mutant γ2 cDNAs were transiently expressed in γ2-null keratinocytes. Transfection of the γ2Δ4 cDNA resulted in restoration of laminin-5 deposition onto the culture substrate, which demonstrates that the γ2 polypeptides carrying a deletion in domain V, upstream of the γ2 proteolytic cleavage site, are assembled into native laminin-5 that is secreted and extracellularly processed. In contrast, transfection of a mutant cDNA expressing the γ2t chain failed to restore laminin-5 immunoreactivity, which indicates that integrity of the γ2 C-terminal amino acid sequences is required for laminin-5 assembly. These results correlate for the first time a functional alteration in a laminin-5 domain with a mild junctional epidermolysis bullosa phenotype. ***Key words: inherited blistering skin diseases/ laminin γ2 chain/molecular genetics. J Invest Dermatol 117:731–739, 2001***

Laminin-5 is a laminin isoform that constitutes the major adhesion ligand of epithelial cells and a putative component of the anchoring filaments, thread-like structures that span the lamina lucida in the basement membrane of the skin and mucous epithelia (Rousselle *et al*, 1991). Laminin-5 is synthesized within the basal epithelial cells as a heterotrimeric molecule composed of an α3 (200 kDa), a β3 (140 kDa) and a γ2 (155 kDa) chain that associate in their C-terminal domains to form a triple-stranded α-helical coiled-coil rod domain (Engel *et al*, 1991). After assembly, laminin-5 molecules are secreted in the extracellular matrix where laminin-5 is found in two forms of 440 and 400 kDa. The 440 kDa trimer is generated by proteolytic cleavage of the α3 chain (Marinkovich *et al*, 1992). Extracellular processing also removes domains IV and V of the laminin γ2 short arm, shortening the γ2 polypeptide from 155 kDa to 105 kDa and reducing the size of laminin-5 to 400 kDa. The role of bone morphogenetic protein 1 in cleaving the γ2 chains in normal human keratinocytes as well as the localization of the cleavage site within the γ2 chain short arm have been recently described (Amano *et al*, 2000).

Laminin-5 has been shown to bind to cells via two integrin receptors, α3β1 and α6β4 (for review see Borradori and Sonnenberg, 1999). The α6β4 integrin is a transmembrane component of hemidesmosomes, the dense cytoplasmic devices that connect the basal cells of stratified and pseudostratified epithelia to the lamina densa through anchoring filaments. The essential role of laminin-5 and α6β4 integrin in epidermal–dermal adhesion has been confirmed by the finding that mutations in either molecule cause junctional epidermolysis bullosa (JEB). JEB is a clinically and genetically heterogeneous group of inherited blistering disorders of skin and mucous membranes, characterized by mesenchymal–epithelial separation within the lamina lucida of the basement membrane zone and by hemidesmosomal abnormalities. On the basis of the clinical severity JEB patients with laminin-5 mutations have been classified into the Herlitz (lethal) and non-Herlitz

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Abbreviations: ASO, allele-specific oligonucleotide; H JEB, Herlitz junctional epidermolysis bullosa; non-H JEB, non-Herlitz junctional epidermolysis bullosa; pAb, polyclonal antibody; PTC, premature termination codon.

(nonlethal) types (Fine *et al*, 2000). The Herlitz variant of JEB (H JEB) is usually associated with mutations resulting in premature termination codons (PTC) in the genes (*LAMA3*, *LAMB3*, *LAMC2*) encoding the three laminin-5 chains. As a result of these PTC mutations, the synthesis of one laminin-5 chain is completely abolished and no functional trimeric laminin-5 is formed, explaining the morphologic abnormalities in hemidesmosome–anchoring filament complexes and the severe defect in epithelial cell adhesion (Aberdam *et al*, 1994; Pulkkinen *et al*, 1994a; Kivirikko *et al*, 1995). On the other hand, several patients are affected with the non-Herlitz form of JEB (non-H JEB), which is associated with missense or exon-skipping mutations in at least one allele of the *LAMB3* and *LAMC2* genes (Pulkkinen *et al*, 1994b, 1998; McGrath *et al*, 1996, 1999; Posteraro *et al*, 1998). The homozygous or heterozygous inheritance of these mutations in the same gene leads to a reduced expression of laminin-5 molecules, which maintain a residual biologic activity (Posteraro *et al*, 1998; Pulkkinen *et al*, 1998; McGrath *et al*, 1999).

In this study we have identified the molecular defect in a child affected with non-H JEB who is a compound heterozygote for two novel *LAMC2* mutations leading to reduced γ2 chain mRNA levels and laminin-5 secretion. Through the expression of the mutant proteins in a γ2-null keratinocyte cell line, we have also assessed the consequences of each mutation on laminin-5 assembly, secretion, and deposition.

MATERIALS AND METHODS

Case report The proband, a 7-y-old male, was the first child of healthy, nonconsanguineous parents. The patient had a history of generalized trauma-induced skin blisters and erosions since birth. Mucosal lesions were limited to transient blisters of the nasal cavity. Nail and tooth dystrophies were also present, but hair was not affected and growth was within normal limits. The diagnosis of JEB was confirmed by ultrastructural examination of a skin biopsy that showed the plane of cleavage to lie within the lamina lucida of the dermal–epidermal junction (DEJ) and the presence of hypoplastic hemidesmosomes (not shown).

Cell cultures Human epidermal keratinocytes were obtained from skin biopsies of the proband and healthy controls and cultivated on a feeder layer of lethally irradiated 3T3-J2 murine fibroblasts (a gift from H. Green, Harvard Medical School, Boston, MA), as described previously (Zambruno *et al*, 1995). For transfection experiments, SV40-immortalized H JEB keratinocytes (LSV5 cell line) that do not express laminin-5 as a consequence of a homozygous nonsense mutation (R95X) in the *LAMC2* gene were used (Miquel *et al*, 1996).

Immunofluorescence studies Frozen sections 5 μm thick were obtained from skin biopsies of the proband and healthy controls and processed for immunofluorescence using a three-step biotin–streptavidin–fluorescein procedure, as described previously (Kanitakis *et al*, 1989). Cultured primary and transfected keratinocytes, grown on glass coverslips in six-well tissue culture plates, were subjected to an indirect immunofluorescence procedure (Gagnoux-Palacios *et al*, 1996). The following monoclonal antibodies (MoAb) and polyclonal antisera (pAb) were used: GB3 (mouse MoAb to laminin-5) (Verrando *et al*, 1991), K140 (mouse MoAb to the laminin-5 β3 chain; gift from R. Burgeson, Cutaneous Biology Research Center, Charlestown, MA) (Marinkovich *et al*, 1992), BM-165 (MoAb to the laminin-5 α3 chain; gift from R. Burgeson) (Rousselle *et al*, 1991), SE85 (rabbit pAb to the laminin-5 α3 chain) (Baudoin *et al*, 1994a), SE144 (rabbit pAb to the laminin-5 γ2 chain) (Vailly *et al*, 1994), 1A8C (mouse MoAb to BPAG2 antigen; gift from K. Owaribe, Nagoya University, Nagoya, Japan) (Owaribe *et al*, 1991), G0H3 (rat MoAb to the α6 integrin subunit; gift from A. Sonnenberg, Netherland Cancer Institute, Amsterdam, The Netherlands) (Sonnenberg *et al*, 1987), and 3E1 (mouse MoAb to the β4 integrin subunit; Telios Pharmaceuticals, San Diego, CA).

Immunoprecipitation analysis Subconfluent keratinocytes were incubated overnight in methionine-cysteine-free medium, in the presence of 100 μCi per ml of [^{35}S] methionine and cysteine (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Cells were detached with 10 mM ethylenediamine tetraacetic acid in phosphate-buffered saline (PBS) pH 7.4 and washed in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells were then lyzed in RIPA buffer (50 mM Tris-HCl, 150 nM NaCl, 1% deoxycolate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.2% sodium azide) pH 7.5 containing protease inhibitors (Complete, Roche Molecular Biochemicals, Mannheim, Germany). Immunoprecipitations of culture medium and cell lysates were carried out by overnight incubation at 4°C of the immunoadsorbents (antibodies adsorbed onto Protein A-Sepharose, Amersham Pharmacia Biotech) with samples of culture medium and cell lysates, followed by extensive washing and elution by boiling in Laemmli sample buffer. Samples were then separated on 6% polyacrylamide gels under reducing conditions, followed by autoradiography. Quantitation of autoradiograms was performed by densitometric scanning with a Gel Doc 1000 (Bio-Rad, Hercules, CA).

Northern analysis Total RNA from primary cultures of epidermal keratinocytes was prepared by the guanidinium-thiocyanate method, as described previously (Chomczynsky and Sacchi, 1987). For each sample, 20 μg of total RNA were separated by electrophoresis through a 1.0% agarose/formaldehyde gel and transferred to Hybond N nylon membrane in 20 × sodium citrate/chloride buffer, as described by the supplier (Amersham Pharmacia Biotech). Membranes were hybridized at high stringency with ^{32}P-labeled probes NA1 (Baudoin *et al*, 1994a), Kal-5.5 (Gerecke *et al*, 1994), and PCR 1.3 (Vailly *et al*, 1994) to detect the mRNA for laminin-5 α3, β3, and γ2 chains, respectively. For loading control, membranes were hybridized with a probe corresponding to the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Quantitation of autoradiograms was performed as described above.

Mutation detection and verification Genomic DNA was extracted from peripheral blood of the proband, the parents, and the controls by standard methods. 100 ng of total DNA were used as a template for amplification of individual exons of *LAMC2* gene, as described previously (Pulkkinen *et al*, 1997). Specifically, to amplify a 205 bp region of the *LAMC2* gene comprising exon 4, primers were (L) 5′ GTTGTGAAGCATTTGGAAGC 3′ and (R) 5′ CTAGTTGGGCAA-GGGACTCT 3′. Primers used for amplification of a 462 bp portion of the *LAMC2* gene containing exon 23 were (L) 5′ AGTTATGGG-TATAGAAGGGC 3′ and (R) 5′ TGACCTGAGCATACCCATTA 3′. Polymerase chain reaction (PCR) amplification products were subjected to heteroduplex analysis, according to the manufacturer's instructions (MDE, FMC Corporation, Rockland, ME). If a heteroduplex band was detected, the PCR product was subcloned and sequenced, as described by Posteraro *et al* (1998). Allele-specific oligonucleotide (ASO) analysis was carried out to verify mutations and to assess their inheritance in the kindred, as described previously (Ruzzi *et al*, 1997). For the exon 23 mutation the ASO used were 5′ CGAGCCAAGACCCAGATCAA 3′ for the wild-type allele and 5′ CGAGCCAAGAACCCAGATCA 3′ for the mutated allele. To verify the exon 4 mutation the ASO were 5′ TCTTCTTCCCCAGAGACT 3′ for the wild-type allele and 5′ CTTCTTCCCCAAAGACTC 3′ for the mutated allele. Nucleotide positions for mutations and polymorphism described in this study are according to *LAMC2* cDNA (Kallunki *et al*, 1992; GenBank accession # Z15008 and Z15009).

Genotype analysis The patient and the parents were genotyped using the following informative genetic markers: HLA DQα (Spinella *et al*, 1997), D7S460 (Hudson *et al*, 1992), ACTBP2 (Polymeropoulos *et al*, 1992), D1S80 (Wirth *et al*, 1993), and HUMTH01 (Edwards *et al*, 1991). For HLA DQα genotyping we used the HLA DQα forensic DNA amplification kit (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ), according to the manufacturer's instructions. An intragenic *LAMC2* polymorphism, 858 C/A in exon 6, discovered in the course of this study was also used for genotype analysis. This polymorphism results in the amino acid substitution D247E and is detected by *Asp*I restriction endonuclease digestion (Roche Molecular Biochemicals) of a 222 bp amplification product containing exon 6 (Pulkkinen *et al*, 1997). The allelic frequency, tested on unrelated healthy individuals (60 chromosomes), was 86% for C and 14% for A, resulting in a value of 0.212 for the polymorphism information content.

Reverse transcriptase PCR (RT-PCR) analysis and cDNA sequencing cDNA was obtained by reverse transcription of total RNA from the patient's cultured keratinocytes, as described by Posteraro *et al* (1998). RT-PCR was carried out using antisense primers that end at the C/A polymorphism detected at nucleotide position 858 and also allow us to distinguish between cDNA molecules derived from the two alleles. For amplification of the paternal transcripts, the primers employed were (L) 5′ GCACCCAAGACCAGAGACTG 3′ (nt 500–519) and (R) 5′ GGAGCCACAAAATAGACAGGT 3′ (nt 858–878). For amplification of maternal transcripts the sense primer was the same as above and

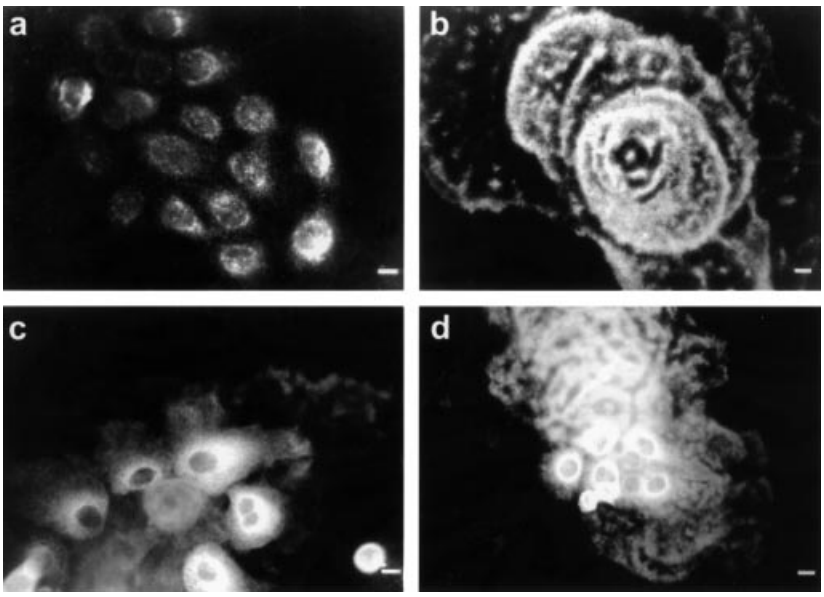

Figure 1. Immunofluorescence localization of laminin-5 in non-H JEB keratinocytes. With antibodies to laminin γ2 chain (*a*, *b*) and heterotrimeric laminin-5 (*c*, *d*), cytoplasmic staining as well as labeling of the extracellular matrix are visible in patient's keratinocytes (*a*, *c*). The intensity of the extracellular staining is strikingly reduced, however, compared to normal control (*b*, *d*). *Bars*: 7 μm (*a*, *b*); 10 μm (*c*, *d*).

the antisense was 5′ GAGCCACAAAATAGACAGGG 3′ (nt 858–877). PCR conditions were: 94°C for 5 min followed by 94°C for 30 s, 65°C for 30 s, 72°C for 30 s (35 cycles), and extension at 72°C for 10 min. The PCR products were subjected to direct sequencing by Thermo Sequenase ^{33}P-radiolabeled terminator cycle method (Amersham Pharmacia Biotech).

To estimate the relative contribution of the two mutant alleles to the reduced level of γ2 chain mRNA detected by northern analysis, RT-PCR amplification combined with *Asp*I (Roche Molecular Biochemicals) restriction enzyme digestion was performed using the following primers: (L) 5′ GCAGCTCTGCAGAATACAGT 3′ (nt 707–726) and (R) 5′ AGATTCCGCAGTAACCTTCG 3′ (nt 1126–1145). The digested PCR products were fractioned by 2% agarose gel electrophoresis.

Plasmids and transient transfection assays Plasmids used in transient transfection assays to express mutated γ2 chains were derived from plasmid pCγ2, which contains the full-length wild-type cDNA encoding the laminin γ2 chain inserted in the expression vector pcDNA3 as previously described (Gagnoux-Palacios *et al*, 1996). This plasmid was used to monitor transfection efficiency. Plasmid pCγ2Δ4 was constructed by replacing the *Aoc*I/*Srf*I (Roche Molecular Biochemicals; Stratagene, La Jolla, CA) fragment of pCγ2 with the corresponding fragment carrying the in-frame skipping of exon 4, obtained by RT-PCR of total RNA from the patient's keratinocytes. To generate the pCγ2t plasmid the *Eco*47III/*Xho*I (Roche Molecular Biochemicals) fragment of pCγ2 was replaced with the corresponding region carrying mutation 3511insA, obtained by RT-PCR of the patient's keratinocyte total RNA. All the expression plasmids were characterized by restriction mapping and sequencing to ensure their identity around cloning sites.

Fifty percent confluent LSV5 monolayers grown in six-well tissue culture plates were transfected using DOSPER liposomal transfection reagent (Roche Molecular Biochemicals), according to the manufacturer's instructions, and subjected to immunofluorescence analysis 48 h later.

RESULTS

Non-H JEB patient's keratinocytes synthesize and secrete a reduced amount of laminin-5 that undergoes processing and deposition into the matrix MoAb GB3, which recognizes a conformational epitope of laminin-5, gave a linear labeling of the DEJ of markedly reduced intensity in the proband's skin compared to the staining of normal control skin. Using antibodies directed against each laminin-5 chain, expression of the laminin γ2 and β3 polypeptides appeared greatly reduced whereas staining for the α3 chain was attenuated (not shown). In contrast, immunoreactivity of the antibodies directed against the other major DEJ components, including α6β4 integrin and BP180, was similar to healthy controls.

Immunofluorescence analysis of patient's cultured keratinocytes revealed laminin-5 staining localized both within the keratinocyte cytoplasm and in the matrix deposited onto the culture vessel. Labeling intensity, however, appeared markedly reduced compared to control keratinocytes (**Fig 1**).

Immunoprecipitation of cultured keratinocyte lysates evidenced that the precursor α3 (200 kDa) and γ2 (155 kDa) laminin-5 chains as well as the β3 (140 kDa) chain are synthesized in the patient's keratinocytes, although in reduced amount compared to normal control keratinocytes (not shown). In addition, immunoprecipitation analysis of spent culture medium of patient's keratinocytes showed the presence of mature laminin-5 as demonstrated by detection of a 165 kDa band (α3 chain) and a 105 kDa band (γ2 chain) (**Fig 2*B***). The immunoprecipitated bands were much fainter than in normal keratinocytes, however, and their intensity was estimated to be about 8% of the normal control by densitometric analysis. Taken together, immunofluorescence and immunoprecipitation results show that residual laminin-5 synthesis, secretion, processing, and deposition into the matrix occur in our non-H JEB patient.

Northern analysis shows markedly reduced levels of γ2 laminin chain mRNA in the non-H JEB patient Northern analysis with a cDNA probe for the laminin-5 γ2 chain resulted in a signal markedly reduced (by about 70%) in the patient compared with a control, after correction of the signal by GAPDH mRNA levels (**Fig 2*A***). In contrast, the signal intensity obtained with probes for the laminin-5 α3 and β3 subunits was comparable to controls (not shown). These findings indicate that *LAMC2* was the candidate gene in the disease.

Mutation analysis reveals compound heterozygosity for a single base insertion and a *de novo* splice site mutation in *LAMC2* Heteroduplex analysis of the PCR products spanning the *LAMC2* gene in the proband's genomic DNA showed two distinct shifts, corresponding to exons 4 and 23. Subcloning and sequencing of the PCR product corresponding to exon 23 detected the insertion of a single base (A) at nucleotide position 3511, leading to a frameshift and resulting in a PTC 110 bp downstream (**Fig 3*A***). Mutation 3511insA predicts a truncated γ2 polypeptide (γ2t) terminating at residue 1168 and therefore lacking the C-terminal 25 amino acids. ASO analysis confirmed the heterozygous state of mutation 3511insA in the proband and demonstrated the maternal inheritance in the kindred (**Fig 3*B***).

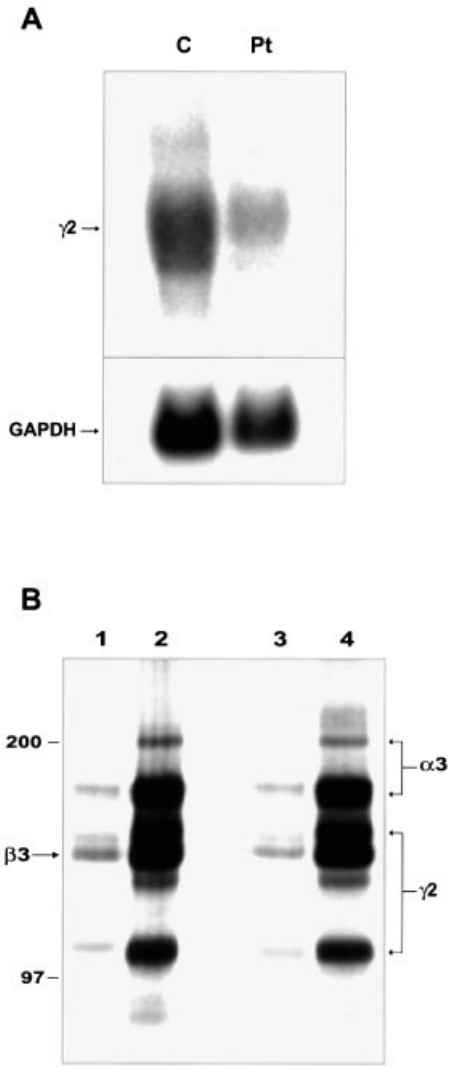

Figure 2. Northern blot and immunoprecipitation analysis of laminin-5. (*A*) Expression of *LAMC2* gene was assessed by northern blot of total RNA purified from cultured patient and normal keratinocytes. Membranes were hybridized with a ^{32}P-labeled *LAMC2* specific probe and, for loading control, the GAPDH probe. The intensity of the signal for the γ2 chain mRNA appears markedly reduced in patient (Pt) compared to control keratinocytes (*C*). (*B*) Cultured keratinocytes from the non-H JEB patient (*lanes 1*, *3*) and a healthy volunteer (*lanes 2*, *4*) were labeled with [^{35}S] methionine and cysteine, and conditioned media were immunoprecipitated with antibodies against laminin-5 β3 (*lanes 3*, *4*) and γ2 chains (*lanes 1*, *2*). The eluates were then analyzed on 6% SDS-PAGE gels under reducing conditions. Protein-bound radioactivity in conditioned media was counted, and equivalent amounts of radioactivity were immunoprecipitated from patient and control media. Highly reduced levels of unprocessed and processed γ2 chain are observed in patient's cells (*1*, *3*) in comparison with normal control keratinocytes (*2*, *4*).

Sequence analysis of the PCR product corresponding to exon 4 and the flanking intronic regions detected a G→A substitution at position −1 of the 3′ splice site of intron 3 (Airenne *et al*, 1996). This mutation was designated 522-1G→A (**Fig 3*C***). ASO analysis confirmed the heterozygous state of the mutation in the proband (**Fig 3*D***). On the other hand, the PCR products from family members, including the father, hybridized only with the wild-type ASO (**Fig 3*D***), suggesting that mutation 522-1G→A occured *de novo* in the paternal allele. Paternity was therefore confirmed by comparison of five informative polymorphic markers (HLA DQα, D7S460, ACTBP2, D1S80, HUMTH01) and the intragenic 858

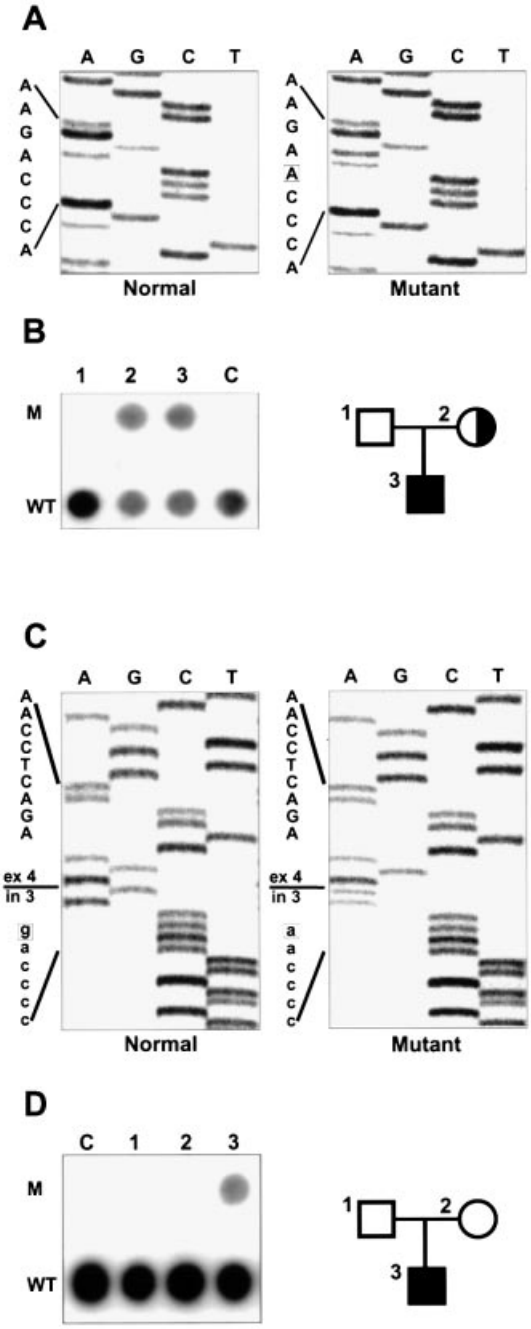

Figure 3. Identification and verification of the heterozygous mutations 3511insA and 522-1G→A in the proband *LAMC2* gene. (*A*) Total genomic DNA was subjected to PCR using primers that amplify a 462 bp region comprising exon 23 of *LAMC2* gene. In comparison with normal control DNA, nucleotide sequencing of patient's DNA reveals a single base insertion (A at position 3511), resulting in a frameshift and a PTC 110 bp downstream. The mutation is designated 3511insA. (*B*) Verification of mutation 3511insA was performed by ASO hybridization of PCR products from the parents, the proband, and a normal control (C). The wild-type (WT) oligomer hybridizes to all DNA samples, whereas the mutant (M) oligonucleotide hybridizes only to DNA from the proband and his mother, indicating that they are heterozygous for mutation 3511insA. (*C*) Total genomic DNA was subjected to PCR using primers that amplify a 205 bp region encompassing *LAMC2* exon 4. Compared with the DNA of a normal control, nucleotide sequencing of non-H JEB patient's DNA reveals a G-to-A transition at the 3′ acceptor splice site of intron 3. The mutation is designated 522-1G→A. (*D*) ASO analysis performed on 205 bp amplimers corresponding to exon 4 with wild-type (WT) and mutated (M) oligomers confirms the heterozygous state of mutation 522-1G→A only in the proband's DNA (3), suggesting the *de novo* occurrence of the mutation in the paternal allele.

C/A polymorphism identified in *LAMC2* in the course of this study. This polymorphism is detected by *Asp*I restriction enzyme digestion. *Asp*I digestion of the PCR product spanning exon 6 (222 bp) revealed that the patient and his father were heterozygous for the C/A polymorphism, whereas the mother was homozygous for the C. These results confirmed that, in the patient, mutation 522-1G→A is brought by the paternal allele. As the mutation is not found in the father's somatic cells, it most probably originated in the father's germ-line cells.

The *de novo* splice site mutation 522-1G→A results in a major mRNA transcript carrying the in-frame skipping of exon 4 As mutation 522-1G→A abolishes the canonical 3′ splice site consensus sequence, an aberrant splicing was expected. mRNA from cultured keratinocytes was therefore analyzed by RT-PCR using antisense oligonucleotides that prime amplification starting from nucleotide position 858 and thus also allow us to differentiate transcripts derived from the two alleles. Analysis on 2% agarose gel of PCR products from the paternal allele (**Fig 4*A***) demonstrated a 279 bp band that results from the in-frame skipping of the exon 4 (99 bp), as confirmed by nucleotide sequencing of the band isolated from the gel (**Fig 4*B***, ***C***). This transcript encodes for a mutated γ2 polypeptide (γ2Δ4) carrying a 33 amino acid deletion within the N-terminal γ2 domain V. In addition, a faint band corresponding in size to a normal splicing product was detected. Isolation and direct sequencing of this minor PCR product identified an abnormal RNA transcript originating from the activation of a cryptic splice site that uses the first two nucleotides (AG) of exon 4 as a new acceptor site (**Fig 4*B***, ***C***). The resulting mRNA transcript carries an out-of-frame deletion of 2 bp and contains a PTC at nucleotide position 534. No mRNA transcripts corresponding to a normal splicing product were identified. Using the primer that anneals to mRNA molecules derived from the maternal allele, only a 378 bp fragment corresponding to a normal splicing product was observed (**Fig 4*B***). The latter finding further confirms that mutation 522-1G→A arose *de novo* on the paternal allele.

Both mutant alleles give rise to unstable transcripts that contribute to the reduced steady-state level of *LAMC2* mRNA We then investigated the relative contribution of the two alleles to the reduced steady-state level of the γ2 chain mRNA detected by northern analysis. RT-PCR of the cDNA region containing the 858 C/A polymorphism (nt 707–1145), followed by *Asp*I digestion, showed that the 286 and 153 bp bands that represent the cleavage products of the maternal cDNA have an intensity similar to the undigested 439 bp band identifying the paternal cDNA fragment (**Fig 4*D***). This result indicates that both mutations affect to a similar degree the decay of the γ2 chain mRNA.

Expression of the mutated γ2 chains in γ2-null keratinocytes: the γ2Δ4 polypeptide rescues laminin-5 secretion and matrix deposition The ability of the two major mutant transcripts to sustain synthesis and secretion of laminin-5 was then investigated by transfecting cDNAs encoding mutant γ2 polypeptides into the cell line LSV5. This keratinocyte cell line is derived from an H JEB patient with a homozygous nonsense mutation (R95X) in the *LAMC2* gene (Miquel *et al*, 1996). LSV5 cells do not synthesize the laminin γ2 chain, but express the laminin α3 and β3 chains; hence transfection of a plasmid (pCγ2) expressing a wild-type γ2 cDNA restores production of functional laminin-5 molecules (Gagnoux-Palacios *et al*, 1996). To assess the biologic activity of the mutated

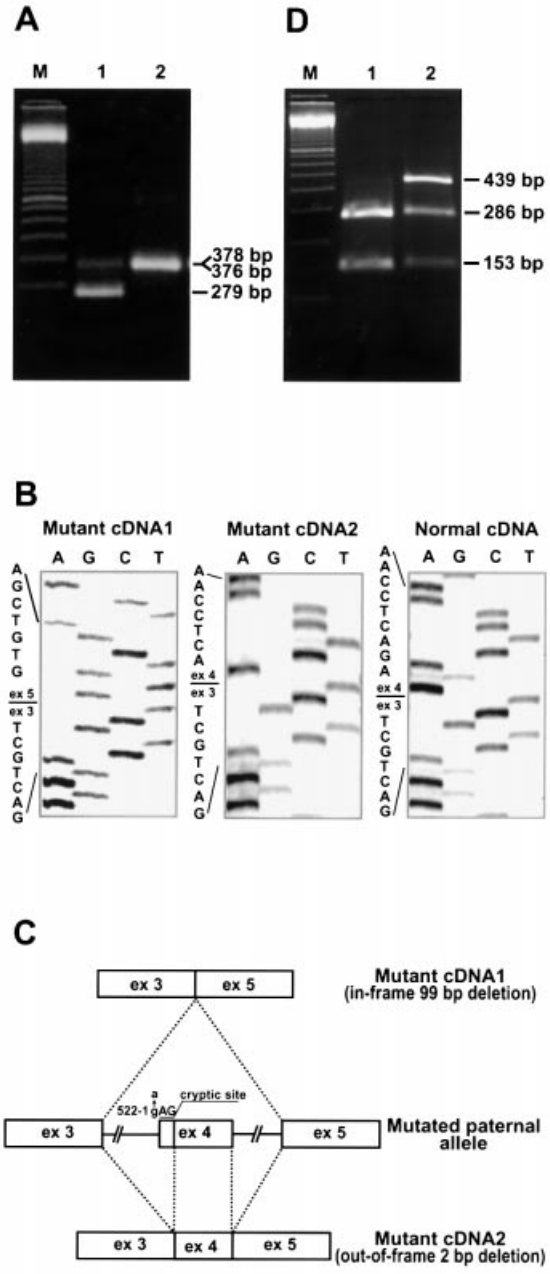

Figure 4. Allele-specific analysis of the transcripts and cDNA sequencing. (*A*) RT-PCR amplification of total RNA obtained from patient's cultured keratinocytes using paternal (*lane 1*) or maternal (*lane 2*) specific antisense oligonucleotides that end at the C/A polymorphism detected at nt 858. Paternal-specific PCR products are represented by two fragments, of 376 and 279 bp. By contrast, a single band of 378 bp is derived from the maternal allele. (*B*) Sequencing of 279 bp fragment shown in *lane 1A* reveals an aberrant mRNA bearing the in-frame skipping of exon 4 (99 bp) (mutant cDNA 1), whereas sequencing of the 376 bp band derived from the paternal allele shows an out-of-frame transcript carrying the deletion of the first two bases of exon 4 (mutant cDNA 2). (*C*) In the middle is schematically represented the genomic structure of the region from exon 3 to exon 5 of the *LAMC2* paternal allele carrying mutation 522-1G→A. The aberrantly spliced transcripts are represented above and below the mutated allele. Splicing events are drawn by dotted lines. The mutation and the cryptic splice site within exon 4 are indicated. (*D*) RT-PCR amplification followed by *Asp*I restriction enzyme digestion was carried out using oligonucleotides spanning the region containing the 858 C/A polymorphism (nt 707–1145) on cDNA from a normal homozygous C/C individual (*lane 1*) and the heterozygous C/A patient (*lane 2*). Two digested bands of 286 and 153 bp are visible in the C/C homozygous subject. In the patient, the maternal transcripts correspond to the same 286 and 153 cleaved bands, whereas the paternal transcripts remain undigested and correspond to the 439 bp band. Note that the intensity of the two maternal bands is similar to that of the paternal one. The markers for molecular size (100 bp DNA ladder, from 100 to 2000 bp) are in *lane M*.

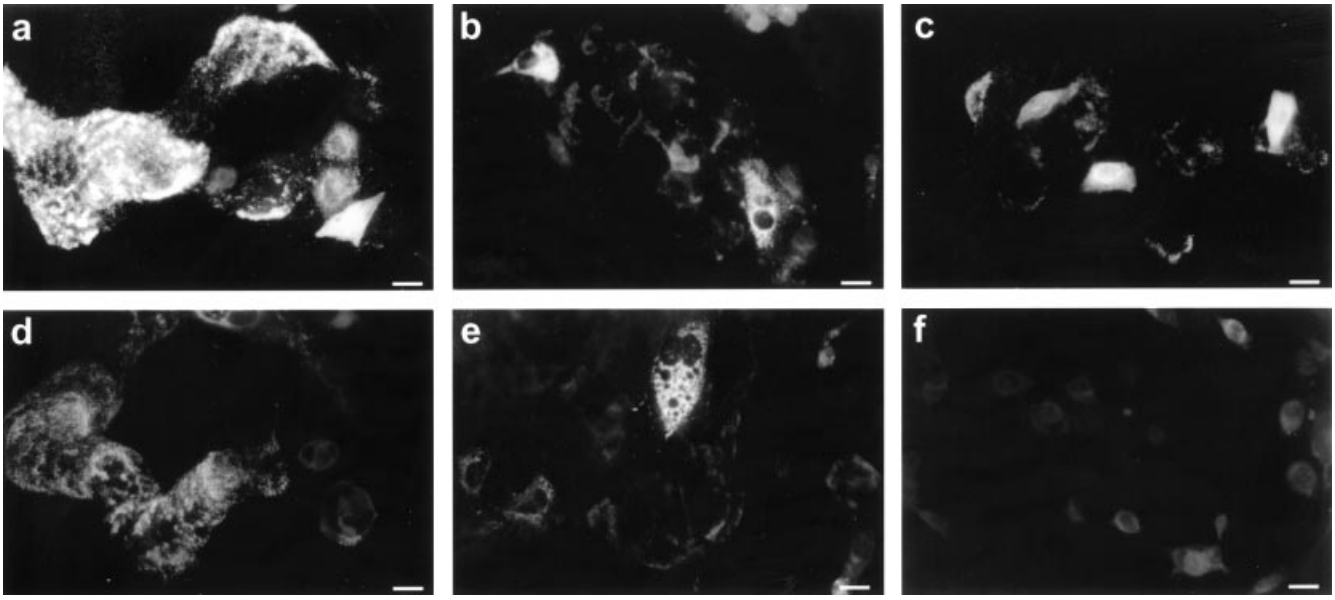

Figure 5. Immunofluorescence staining of γ2-null LSV5 cells transiently expressing the wild-type and mutated γ2 chains. Forty-eight hours after transfection, cells were stained with antibodies to laminin γ2 chain (*a*, *b*, *c*) and to heterotrimeric laminin-5 (*d*, *e*, *f*). In LSV5 cells the γ2 chain and, consequently, laminin-5 molecules are absent (Miquel *et al*, 1996), but reappear after transfection with the wild-type γ2 cDNA resulting in strong intracytoplasmic and extracellular labeling (*a*, *d*). LSV5 cells transfected with plasmid pCγ2Δ4 to express the exon-4-deleted γ2 chain show an intense cytoplasmic labeling and a fainter extracellular staining with both antibodies used (*b*, *e*). LSV5 cells transfected with plasmid pCγ2t to express the C-terminal truncated γ2 chain show immunostaining with anti-γ2 antibody (*c*) but no reactivity with anti-laminin-5 antibody (*f*). Transfection experiments were repeated three times with similar results. *Scale bar*: 12 μm.

γ2Δ4 and γ2t chains, LSV5 cells were transfected with the eucaryotic expression vectors pCγ2Δ4 and pCγ2t. Expression of the transfected cDNAs was monitored by immunofluorescence analysis using pAb SE144 specific to the laminin γ2 chain, and MoAb GB3 specific to the native laminin-5 molecule. In all LSV5 transfected cells, pAb SE144 labeled the keratinocyte cytoplasm and the matrix deposited by the cells on the plastic culture vessel, confirming that both mutant γ2 polypeptides are actively synthesized and secreted (**Fig 5**). The extracellular staining appeared less intense in LSV5 cells transfected with either mutant γ2 polypeptide, however, than in those transfected with the control pCγ2 plasmid. Using MoAb GB3, LSV5 cells transfected with plasmid pCγ2Δ4 showed a labeling of both the cytoplasm and extracellular matrix (**Fig 5**). These observations indicate that mutant γ2 polypeptides with an internal deletion in the EGF-like repeat 3 of domain V assemble into trimeric laminin-5 molecules that are secreted and incorporated into the matrix. Consistent with the results obtained with pAb SE144, the staining was less intense in the γ2Δ4 cells than in cells transfected with the control plasmid. In contrast, the cells transfected with the pCγ2t plasmid were not reactive to MoAb GB3, which suggests that the mutant γ2t chain is not assembled into trimeric laminin-5 (**Fig 5**).

DISCUSSION

In this report we describe the molecular defect in a child suffering from the non-H variant of JEB. Mutation analysis of the *LAMC2* gene showed that our proband is a compound heterozygote for two novel mutations, the *de novo* splice site mutation 522-1G→A and the insertion mutation 3511insA, which further extend the molecular heterogeneity of JEB.

The maternally inherited single base insertion 3511insA leads to a frameshift spanning 37 amino acids and a downstream PTC in exon 23 of *LAMC2* gene, 25 amino acids upstream of the physiologic termination codon. It is well established that PTC generated by genetic defects in laminin-5 genes as well as in other genes cause decay of the aberrant mRNA transcripts leading to undetectable gene products (Aberdam *et al*, 1994; Baudoin *et al*, 1994b; Matsui *et al*, 1998; for review see Maquat, 1995). On the other hand, it has been shown that mRNA decay is less pronounced when a PTC localizes in the last exon of the mutated gene (Cheng *et al*, 1990; Hall and Thein, 1994; for review see Hentze and Kulozik, 1999). Consistent with this observation, northern blot and allele-specific PCR analysis of the RNA transcripts isolated from our patient detected significant amounts of mRNA carrying mutation 3511insA that encodes γ2 polypeptides (γ2t) harboring a C-terminal stretch of 37 aberrant amino acids and truncated of the last 25 residues.

The paternal allele mutation 522-1G→A was shown to have arisen as a *de novo* event, probably reflecting a germ-line mosaicism in the father, which bears implications for genetic counseling regarding the risk of recurrence in subsequent offspring. Mutation 522-1G→A affects the acceptor splice site of intron 3, alters the correct splicing of *LAMC2* pre-mRNA, and generates two mutated transcripts. The major messenger RNA bears an in-frame deletion of 99 nucleotides (transcript 1) whereas the less abundant transcript (transcript 2) carries a two-nucleotide deletion causing a shift of the reading frame. Transcript 2 results from the activation of a cryptic splice site formed by the first two nucleotides of exon 4 and carries a PTC at nucleotide 534 that is expected to cause active mRNA decay. The more abundant transcript 1 carries the in-frame skipping of exon 4 and codes for a γ2 polypeptide (γ2Δ4) lacking 33 amino acids within the γ2 domain V, upstream of the proteolytic cleavage site of the chain. Exon-skipping leading to synthesis of internally deleted proteins that maintain a residual activity has been reported in a number of genes, including those for laminin-5 (Pulkkinen *et al*, 1994b, 1998; McGrath *et al*, 1996, 1999; Posteraro *et al*, 1998). Specifically, a homozygous splice site mutation leading to the in-frame skipping of *LAMC2* exon 9 has been described in two siblings affected with non-H JEB (Pulkkinen *et al*, 1994b).

The genetic studies performed in our patient showed that both mutations are responsible for the reduced steady-state levels of *LAMC2* mRNA detected by northern analysis. The genetic studies could not provide information on the contribution of each mutation to the mild JEB phenotype, however, as both mutant

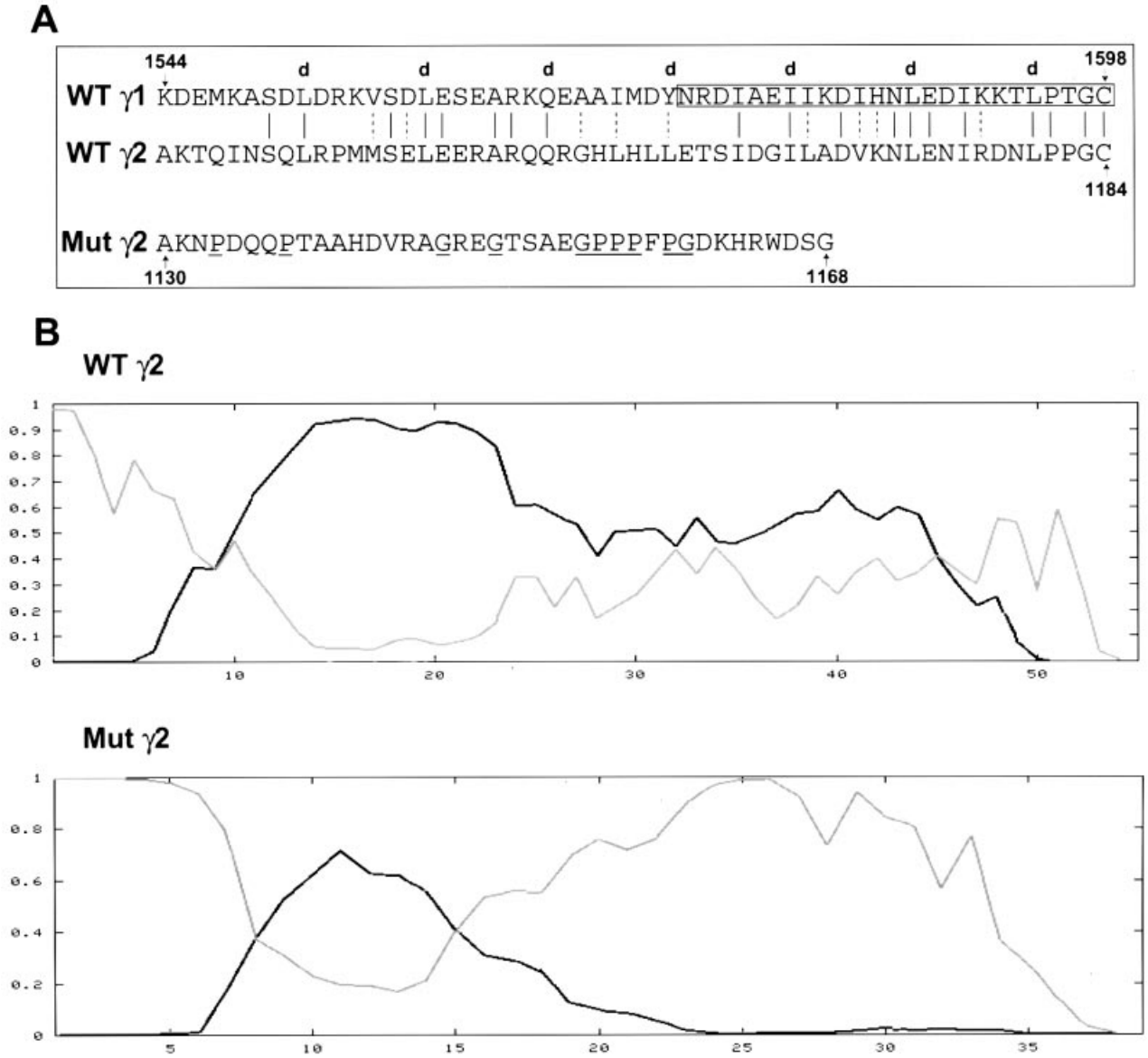

Figure 6. Effect of frameshift mutation 3511insA on protein sequence and secondary structure. (*A*) Alignment and conservation between the C-terminal sequences of wild-type murine γ1 (Swissprot accession No. P02468) and human γ2 chains. The mutant γ2 chain with the aberrant amino acid sequence generated by the frameshift mutation 3511insA and starting from residue 1132 is also shown. The 'd' positions in the heptad repeat of laminin long arm is indicated above the sequences. The γ1 sequences required for dimer and trimer assembly are boxed. The mutant chain terminates prematurely at residue 1168. New proline and glycine residues in the mutant sequence that are known to act as α-helix breakers are underlined. (*B*) Secondary structure prediction plots according to Garnier *et al* (1996). The normal sequence shows propensity for α-helix (WT γ2). By contrast, the mutant sequence shows a severe disruption of the α-helix acquiring a random coil profile (Mut γ2). *Black*, α-helix; *gray*, random coil.

γ2t and γ2Δ4 chains might potentially retain a biologic activity. The respective effect that the mutant γ2t and γ2Δ4 chains have on laminin-5 synthesis and secretion was therefore assessed in transient transfection assays of γ2-null LSV5 cells using mutant γ2 cDNAs. The finding that the mutant γ2t chains do not incorporate into laminin-5 molecules is in keeping with the observation that the C-terminal regions of the laminin chains are critical for assembly of the heterotrimers. As for the other laminin isoforms, formation of a stable β3γ2 heterodimer is the initial step in assembly of laminin-5 (Matsui *et al*, 1995). According to the model proposed by Beck *et al* (1993), laminin chain assembly requires formation of a rod-like triple-stranded α-helical coiled-coil C-terminal domain. In this structure, the interacting edges of the three chains are mostly formed by hydrophobic residues in position a and d of an $(abcdefg)_n$ heptad repeat, and by a distinct pattern of charged residues in positions e and g. In addition, evidence has been provided on the critical role of short sequences (less than 100 amino acid residues) at the C-terminal end of each laminin chain for triple-stranded coiled-coil structure formation (Engel *et al*, 1991; Hunter *et al*, 1992; Nomizu *et al*, 1994, 1996; Utani *et al*, 1994; Antonsson *et al*, 1995; Kammerer *et al*, 1995). As shown in **Fig 6**, the frameshift mutation 3511insA generates several proline and glycine residues that interrupt the heptad sequence repeat and hamper the α-helicity and the ionic interactions of the coiled-coil domain I (Garnier *et al*, 1996). In addition, the truncated γ2t chain lacks the 25 C-terminal residues that include the sequence DVKNLE that is conserved in the γ1 and γ2 laminin chains and is required for heterotrimerization in laminin 1 and laminin 2 (**Fig 6*A***) (Utani *et al*, 1994). The cysteine residue (Cys1184) involved in the C-terminal disulfide interchain bond is also missing (Antonsson *et al*, 1995).

Conversely, transient transfection assays showed that the mutant γ2Δ4 chains incorporate into heterotrimeric laminin-5 molecules

that are secreted and deposited onto the cell culture substrate. These results suggest that the immunoreactive laminin-5 detected at the DEJ of our patient corresponds to molecules harboring the mutated γ2Δ4 chain. We have recently shown that the unprocessed form of the γ2 chain (comprising the short arm domain IV and V) drives incorporation of laminin-5 into the extracellular matrix secreted by LSV5-transfected keratinocytes (Gagnoux-Palacios *et al*, 2001). Mutation analysis based on transfer of mutant γ2 cDNAs into γ2-null LSV5 keratinocytes also demonstrated that deletions in the γ2 short arm globular domain IV hamper the deposition of laminin-5, whereas laminin-5 lacking the epidermal-growth-factor-like repeat 2 and 3 of the γ2 domain V is efficiently layered down (Gagnoux-Palacios *et al*, 2001). The findings in our non-H JEB patient are in keeping with these observations and confirm that the γ2 chain domain V is not essential to the extracellular secretion and deposition of laminin-5 to the basement membrane.

Amino acid substitutions generated by site-directed mutagenesis in the γ2 domain IV, however, may affect the adhesive function of laminin-5 without limiting the deposition of the protein, which underscores the crucial role of the γ2 chain short arm in cell adhesion (Gagnoux-Palacios *et al*, 2001). Considering that in our patient the laminin-5 molecules with a mutated γ2Δ4 chain are correctly processed and layered down, it is tempting to hypothesize that the deletion of exon 4 within the γ2 domain V negatively affects the adhesive functions of laminin-5 and causes non-H JEB. Due to both mRNA decay and defective assembly of the γ2t chain, however, the mutated laminin-5 molecules are secreted in reduced amounts by the patient's keratinocytes. Therefore, the reduced availability of mutated laminin-5 molecules may destabilize the basement membrane structure and sensibly enhance the effect of the mutated γ2Δ4 polypeptide on cell adhesion. Thus, the pathologic phenotype observed in our non-H JEB patient probably results from the combined effect of a reduced synthesis and an altered functionality of laminin-5.

In conclusion, our findings disclose the complex effect that genetic mutations have in non-H JEB patients that constitute potential candidates to a gene therapy approach of the condition. Our results also contribute to a better understanding of the physiologic functions of the domains of laminin-5.

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REFERENCES

Aberdam D, Galliano MF, Vailly J, *et al:* Herlitz's junctional epidermolysis bullosa is genetically linked to mutations in the nicein/kalinin (laminin-5) LAMC2 gene. *Nat Genet* 6:299–304, 1994

Airenne T, Haakana H, Sainio K, Kallunki T, Kallunki P, Sariola H, Tryggvason K: Structure of the human laminin γ2 chain gene (*LAMC2*): alternative splicing with different tissue distribution of two transcripts. *Genomics* 32:54–64, 1996

Amano S, Scott IC, Takahara K, *et al:* Bone morphogenetic protein 1 (BMP-1) is an extracellular processing enzyme of the laminin-5 γ2 chain. *J Biol Chem* 275:22728–22735, 2000

Antonsson P, Kammerer RA, Schulthess T, Hänisch G, Engel J: Stabilization of the α-helical coiled-coil domain in laminin by C-terminal disulfide bonds. *J Mol Biol* 250:74–79, 1995

Baudoin C, Miquel C, Blanchet-Bardon C, Gambini C, Meneguzzi G, Ortonne J-P: Herlitz junctional epidermolysis bullosa keratinocytes display heterogeneous defects of nicein/kalinin gene expression. *J Clin Invest* 93:862–869, 1994a

Baudoin C, Miquel C, Gagnoux-Palacios L, *et al:* A novel homozygous nonsense mutation in the *LAMC2* gene in patients with the Herlitz junctional epidermolysis bullosa. *Hum Mol Genet* 3:1909–1910, 1994b

Beck K, Dixon TW, Engel J, Parry DAD: Ionic interaction in the coiled-coil domain of laminin determine the specificity of chain assembly. *J Biol Chem* 231:311–323, 1993

Borradori L, Sonnenberg A: Structure and function of hemidesmosomes: more than simple adhesion complexes. *J Invest Dermatol* 112:411–418, 1999

Cheng J, Fogel-Petrovic M, Maquat LE: Translation to near the distal end of the penultimate exon is required for normal levels of spliced triosephosphate isomerase mRNA. *Mol Cell Biol* 10:5215–5225, 1990

Chomczynsky P, Sacchi N: Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987

Edwards A, Civitello A, Hammond HA, Caskey CT: DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am J Hum Genet* 49:746–756, 1991

Engel J, Hunter I, Schulthess T, Beck K, Dixon TW, Parry DAD: Assembly of laminin isoforms by triple and double stranded coiled-coil structures. *Biochem Soc Trans* 19:839–844, 1991

Fine JD, Eady RAJ, Bauer EA, *et al:* Revised classification system for inherited epidermolysis bullosa: report of the Second International Consensus Meeting on diagnosis and classification of epidermolysis bullosa. *J Am Acad Dermatol* 42:1051–1066, 2000

Gagnoux-Palacios L, Vailly J, Durand-Clement M, Wagner E, Ortonne J-P, Meneguzzi G: Functional re-expression of laminin-5 in laminin-γ2-deficient human keratinocytes modifies cell morphology, motility, and adhesion. *J Biol Chem* 271:18437–18444, 1996

Gagnoux-Palacios L, Allegra M, Spirito F, Pommeret O, Romero C, Ortonne J-P, Meneguzzi G: The short arm of the laminin γ2 chain plays a pivotal role in the incorporation of laminin-5 into the extracellular matrix and in cell adhesion. *J Cell Biol* 153:835–849, 2001

Garnier J, Gibrat J-F, Robson B: GOR method for predicting protein secondary structure from amino acid sequence. *Method Enzymol* 266:540–553, 1996

Gerecke D, Wagman DW, Champliaud MF, Burgeson RE: The complete primary structure for a novel laminin chain, the laminin B1k chain. *J Biol Chem* 269:11073–11080, 1994

Hall GW, Thein S: Nonsense codon mutations in the terminal exon of the β-globin gene are not associated with a reduction in β-mRNA accumulation: a mechanism for the phenotype of dominant β-thalassemia. *Blood* 83:2031–2037, 1994

Hentze MW, Kulozik AE: A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* 96:307–310, 1999

Hudson TJ, Engelstein M, Lee MK, *et al:* Isolation and chromosomal assignment of 100 highly informative human simple sequence repeat polymorphisms. *Genomics* 13:622–629, 1992

Hunter I, Schulthess T, Engel J: Laminin chain assembly by triple and double stranded coiled-coil structures. *J Biol Chem* 267:6006–6011, 1992

Kallunki P, Sainio K, Eddy R, *et al:* A truncated laminin chain homologous to the B2 chain: structure, spatial expression, and chromosomal assignment. *J Cell Biol* 119:679–693, 1992

Kammerer RA, Antonsson P, Schulthess T, Fauser C, Engel J: Selective chain recognition in the C-terminal α-helical coiled-coil region of laminin. *J Mol Biol* 250:64–73, 1995

Kanitakis J, Zambruno G, Viac J, Tommaselli L, Thivolet J: Expression of an estrogen receptor-associated protein (p29) in epithelial tumors of the skin. *J Cutan Pathol* 16:272–276, 1989

Kivirikko S, McGrath JA, Baudoin C, *et al:* A homozygous nonsense mutation in the α3 chain gene of laminin-5 (*LAMA3*) in lethal (Herlitz) junctional epidermolysis bullosa. *Hum Mol Genet* 4:959–962, 1995

Maquat LE: When cells stop making sense: effect of nonsense codons on RNA metabolism in vertebrate cells. *RNA* 1:453–465, 1995

Marinkovich MP, Lunstrum GP, Burgeson RE: The anchoring filament protein kalinin is synthesized and secreted as a high molecular weight precursor. *J Biol Chem* 267:17900–17906, 1992

Matsui C, Wang CK, Nelson CF, Bauer EA, Hoeffler WK: The assembly of laminin-5 subunits. *J Biol Chem* 270:23496–23503, 1995

Matsui C, Pereira P, Wang CK, *et al:* Extent of laminin-5 assembly and secretion effect junctional epidermolysis bullosa phenotype. *J Exp Med* 187:1273–1283, 1998

McGrath JA, Christiano AM, Pulkkinen L, Eady RA, Uitto J: Compound heterozygosity for nonsense and missense mutations in the *LAMB3* gene in nonlethal junctional epidermolysis bullosa. *J Invest Dermatol* 106:1157–1159, 1996

McGrath JA, Ashton GHS, Mellerio JE, Salas-Alanis JC, Swensson O, McMillan JR, Eady RAJ: Moderation of phenotype severity in dystrophic and junctional forms of epidermolysis bullosa through in-frame skipping of exons containing non-sense or frameshift mutations. *J Invest Dermatol* 113:314–321, 1999

Miquel C, Gagnoux-Palacios L, Durand-Clement M, Marinkovich P, Ortonne J-P, Meneguzzi G: Establishment and characterization of cell line LSV5 that retains the altered adhesive properties of human junctional epidermolysis bullosa keratinocytes. *Exp Cell Res* 224:279–290, 1996

Nomizu M, Otaka A, Utani A, Roller PP, Yamada Y: Assembly of synthetic laminin peptides into triple-stranded coiled-coil structure. *J Biol Chem* 269:30386–30392, 1994

Nomizu M, Utani A, Beck K, Otaka A, Roller PP, Yamada Y: Mechanism of laminin chain assembly into a triple-stranded coiled-coil structure. *Biochemistry* 35:2885–2893, 1996

Owaribe K, Nishizawa Y, Franke WW: Isolation and characterization of hemidesmosomes from bovine corneal epithelial cells. *Exp Cell Res* 192:622–630, 1991

Polymeropoulos MH, Rath DS, Xiao H, Merril CR: Tetranucleotide repeat polymorphism at the human beta-actin related pseudogene H-beta-Ac-psi-2 (ACTBP2). *Nucl Acids Res* 20:1432, 1992

Posteraro P, Sorvillo S, Gagnoux-Palacios L, *et al:* Compound heterozygosity for an out-of-frame deletion and a splice site mutation in the *LAMB3* gene causes

nonlethal junctional epidermolysis bullosa. *Biochem Bioph Res Commun* 243:758–764, 1998

Pulkkinen L, Christiano AM, Gerecke DR, Wagman DW, Burgeson RE, Pittelkow MR, Uitto J: A homozygous nonsense mutation in the β3 chain of laminin-5 (*LAMB3*) in Herlitz junctional epidermolysis bullosa. *Genomics* 24:357–360, 1994a

Pulkkinen L, Christiano AM, Airenne T, Haakana H, Tryggvason K, Uitto J: Mutations in the γ2 chain gene (*LAMC2*) of kalinin/laminin-5 in the junctional forms of epidermolysis bullosa. *Nat Genet* 6:293–297, 1994b

Pulkkinen L, McGrath J, Airenne T, *et al:* Detection of novel *LAMC2* mutations in Herlitz junctional epidermolysis bullosa. *Mol Med* 3:122–133, 1997

Pulkkinen L, Jonkman MF, McGrath JE, Kuijpers A, Paller AS, Uitto J: *LAMB3* mutations in generalized atrophic benign epidermolysis bullosa: consequences at the mRNA and protein levels. *Lab Invest* 78:859–867, 1998

Rousselle P, Lunstrum GP, Keene DR, Burgeson RE: Kalinin an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J Cell Biol* 114:567–576, 1991

Ruzzi L, Gagnoux-Palacios L, Pinola M, Belli S, Meneguzzi G, D'Alessio M, Zambruno G: A homozygous mutation in the α6 gene in junctional epidermolysis bullosa with pyloris atresia. *J Clin Invest* 99:2826–2831, 1997

Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J: A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. *J Biol Chem* 262:10376–10383, 1987

Spinella A, Marsala P, Biondo R, Montagna P: Italian population allele and genotype frequencies for the AmpliType PM and the HLA-DQ-alpha loci. *J Forensic Sci* 42:514–518, 1997

Utani A, Nomizu M, Timpl R, Roller PP, Yamada Y: Laminin chain assembly. Specific sequences at the C terminus of the long arm are required for the formation of specific double- and triple-stranded coiled-coil structures. *J Biol Chem* 269:19167–19175, 1994

Vailly J, Verrando P, Champliaud MF, *et al:* The 100-KDA chain of nicein/kalinin is a laminin B2 chain variant. *Eur J Biochem* 219:209–218, 1994

Verrando P, Blanchet-Bardon C, Pisani A, *et al:* Monoclonal antibody GB3 defines a widespread defect of several basement membranes and a keratinocyte dysfunction in patients with lethal junctional epidermolysis bullosa. *Lab Invest* 64:85–92, 1991

Wirth B, Voosen B, Rohrig D, Knapp M, Piechaczek B, Rudnik-Schoneborn S, Zerres K: Fine mapping and narrowing of the genetic interval of the spinal muscular atrophy region by linkage studies. *Genomics* 15:113–118, 1993

Zambruno G, Marchisio PC, Marconi A, Vaschieri C, Melchiori A, De Giannetti A, Luca M: Transforming growth factor-β1 modulates β1 and β5 integrin receptors and induces the *de novo* expression of the αvβ6 heterodimer in normal human keratinocytes: implications for wound healing. *J Cell Biol* 129:853–865, 1995